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AEROSOL TRANSMISSION OF HANTAAN AND RELATED VIRUSES TO LABORATORY RATS

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Abstract. Hantaan, Seoul, and Puumala viruses were transmitted to 12–16-week-old female Wistar *Rattus norvegicus* by inhalation. The rodent infectious dose for each virus by intramuscular inoculation and by inhalation was determined, as was the infectious dose for Vero E-6 cells by direct plaque assay.

Hantaan (HTN), Seoul (SEO), and Puumala (PUU) viruses are members of the family Bunyaviridae, in the proposed genus *Hantavirus*.[1] They are responsible for human disease ranging in severity from subclinical to hemorrhagic disorders collectively referred to as hemorrhagic fever with renal syndrome (HFRS).[2] Natural hosts for HTN, PUU, and SEO viruses are striped field mice, *Apodemus agrarius*, bank voles, *Clethrionomys glareo[illegible]*,[3,4] and domestic rats, *Rattus norvegicus*,[4] respectively.

Previous studies suggest that aerosolized virus may be an important route of transmission for hantaviruses.[5–7] Park[8] and Lee et al.[5] showed intercage transmission of HTN virus between cages of *Apodemus* sp. 1–4 m apart. Isolation of HTN[5] virus and PUU[7] virus from feces, urine, and saliva implied that environmental contamination and subsequent virus aerosolization may occur. Yanagihara et al.[7] showed horizontal intracage transmission of PUU virus that coincided with virus shedding in oropharyngeal secretions and was compatible with transmission via grooming among bank voles. Additionally, aerosolized HTN[9] and SEO[10] viruses were suspected as the source of infections among laboratory workers. Epidemiological studies by Xu et al.[11] provided further circumstantial evidence that airborne transmission occurs by revealing higher infection rates among workers who performed heavy farm work and slept on the ground than those who did not. Presumably these workers were at greater risk by inhalation of aerosolized virus in dust. Although Lee et al.[5] explored *Apodemus* susceptibility to HTN viral infection after different routes of inoculation, aerosol exposure was not evaluated. Consequently, there is little direct evidence that establishes aerosol transmission of these viruses. This study was designed to determine the possibility of aerosol transmission of 3 hantaviruses to laboratory rats. Additionally, we compared rat sensitivity to intramuscular (im) and aerosol routes of infection.

MATERIALS AND METHODS

Virus and rodent history

Viruses used were the prototype HTN (strain 76-118) virus isolated in 1976,[12] SEO (strain HR80-39) virus isolated in 1982,[4,13] and PUU (strain 83-223L) virus isolated in 1984.[3] HTN virus had undergone 5 passages in *Apodemus*, 3 passages in A549 cells,[14] and 4 passages in Vero E-6 (American Type Culture Collection No. CRL 1586, Rockville, Maryland) cells. The SEO isolate had been passed in Wistar rats 3 times and 10 times in Vero E-6 cells. PUU virus was passed in Vero E-6 cells 8 times.

Female outbred Wistar Crl:(WR)BR rats (Charles River Laboratories, Wilmington, Massachusetts) were exposed at 12–16 weeks of age and ranged in weight from 200–290 g. Wistar rats were previously shown to be susceptible to hantaviruses after im inoculation.[1,6,15]

Experimental design

Hantavirus-seronegative rats, as determined by immunofluorescent antibody (IFA) assay, were exposed im or by aerosol to 1 of 3 hantaviruses and examined for seroconversion by IFA assay 28–30 days post-infection. Rats with positive

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post-exposure titers (IFA ≥ 32) were considered to have seroconverted due to viral infection. The 30-day incubation period was based on previous studies showing that virus first appeared in excreta 9 days post-infection,[5] and that measurable antibody to hantaviruses appeared at approximately day 18.[6] Lee et al.[16] found that intracage infection did not occur among Fischer rats exposed for 7 days to rats that were infected with HTN virus 28 days previously. Therefore, 28 days provided adequate time for antibody development, while minimizing the potential for detecting antibody due to secondary infections.

Animal controls

Each aerosol and im exposure trial included 5–7 groups of rats exposed to varying virus concentrations and a negative control group exposed to diluent only. All experiments were performed in duplicate. To verify that intracage transmission did not lead to antibody production prior to post-exposure bleeding, 1 unexposed rat was placed in each cage of rats after exposure of the 2 groups receiving the highest virus concentrations.

Serological techniques and serum collection

Hantaviral antibody was measured by IFA assay, and titers of representative sera were confirmed by plaque reduction neutralization (PRN) tests as described previously.[17] Briefly, serial 4-fold dilutions of rat sera were tested by IFA assay with Vero E-6 cell spot slides infected with Hantaan virus, strain 76-118. A fluoroisothiocyanate (FITC)-labeled anti-rat IgG conjugate (Cooper Biomedical, Malvern, Pennsylvania) was used, and titers were recorded as the highest serum dilution yielding characteristic cytoplasmic fluorescence. Median infectious doses (ID_{50}) and graphical data illustrations were accomplished by Statistical Analysis System (SAS) probit analysis and SAS graph, respectively.

PRN tests were conducted in duplicate by mixing equal volumes of diluted serum with 200 plaque forming units (PFU) of each hantavirus strain, incubating them overnight at 4°C, and then inoculating 0.2 ml into 25 cm^2 flasks of Vero E-6 cells. A 1% agarose overlay was applied after a 1 hr adsorption period and flasks were stained with a neutral red-agarose solution after 10–14 days of incubation at 37°C. Plaques were counted 24–48 hr later. Neutralization titers were recorded as a reciprocal of the highest serum dilution neutralizing ≥80% of the plaque dose.

Ketamine HCl and xylazine were used to provide analgesia and restraint for blood sample collection and for placement of rats in the aerosol exposure apparatus. Anesthesia was accomplished by im injection of a 0.2 ml mixture of xylazine (4.6 mg/ml) and ketamine HCl (76.9 mg/ml). Pre- and post-exposure blood samples were collected by cardiac puncture.

Confirmation of infection

To confirm that seroconversions were due to viral replication rather than aerosol immunization with inactivated virus, 2 groups of 8 and 15 rats each were inoculated im with different concentrations of inactivated HTN virus. The resulting antibody titers were compared to titers elicited by live virus inoculation (im). Viral inactivation was accomplished by exposing virus to 0.4% β-propiolactone (BPL) (Oneal, Jones, and Feldman, St. Louis, Missouri) for 96 hr at 4°C and then to radiation with 2×10^6 roentgens in a Gammacell 220 Cobalt 60 irradiator.

Aerosol exposure

Small-particle aerosols with a mass median aerodynamic diameter of 1.3–1.5 μm and a geometric standard deviation of 1.85 of a log-normal distribution were produced with a Collison nebulizer[18] at 22°C and atmospheric pressure using serially diluted virus in a dynamic, nose only exposure system. The dilution medium was Eagle's minimum essential medium containing 10% (v/v) fetal bovine serum (FBS), 0.2 mM nonessential amino acids, 8 mM L-glutamine, 762 U/ml penicillin, 0.19 mg/ml streptomycin, and 0.7 μg/ml fungizone. Groups of 7–8 anesthetized rats were exposed for 10 min. Respiratory minute volumes were estimated with Guyton's formula,[19] then corrected for the 30% decrement in the rat's respiratory rate induced by anesthesia. A 5-min aerosol sample was collected in an all-glass impinger (AGI) containing 20 ml of the above medium supplemented with 5% FBS and 0.1% (v/v) Antifoam Y-30 Emulsion (Dow-Corning, Midland, Michigan). This sample was used to determine the aerosol viral titer by direct plaque assay and for viral isolation on Vero E-6 cells after procedures described previously.[17] Total in-

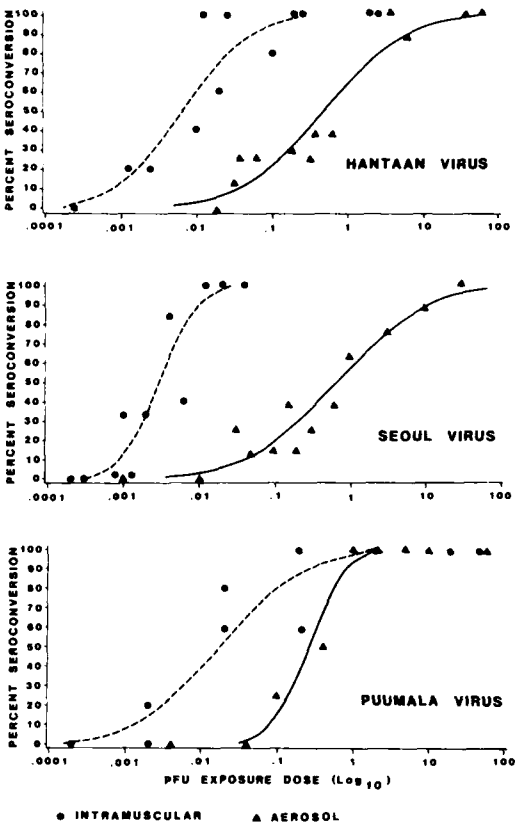

FIGURE 1. Acquisition of IFA antibody among Wistar rats after intramuscular (●) or aerosol (▲) exposure to Hantaan, Seoul, or Puumala virus at 28–30 days post-exposure.

fectious inhaled doses of virus were calculated from the corrected minute volumes and the aerosol concentration of virus delivered.

Intramuscular exposure

Intramuscular inoculation of rats provided positive controls for each aerosol exposure and a means of comparing relative sensitivity of rats to both routes of infection. Virus concentrations administered im were lower, relative to aerosol exposure doses, to obtain im dose-response curves. Rats were separated into groups of 4–6 animals and injected im with 0.5 ml of serially diluted virus. Inocula were titrated by direct plaque assay and cocultivated on Vero E-6 cells for dose determination and virus isolation.

RESULTS

Rats were found to be highly susceptible to each hantavirus after aerosol exposure (Fig. 1); however, they were at least 18 times more sensitive to the same viral dose administered im. As determined from the HTN virus dose-response curves, the ID_{50} for aerosol and im exposures were 0.5 and 0.007 PFU, respectively. Similar response curves were observed for SEO and PUU viruses, and further illustrate the sensitivity of Wistar rats to these hantaviruses by either route of infection.

The rats' PFU ID_{50} for each virus by both routes of exposure are shown in Table 1. Aerosol and im ID_{50} values for SEO virus were 0.7 PFU and 0.003 PFU, while the PUU viral aerosol and im ID_{50} were 0.3 and 0.016 PFU, respectively. For both routes of exposure, the ID_{50} and response curves for HTN virus were intermediate between those for SEO and PUU viruses. The ID_{50} confidence intervals for all 3 viruses overlapped for each exposure route.

Figure 1 also shows that rats were consistently infected by inocula in which virus was diluted beyond plaque assay detection limits. Additionally, isolation of virus from inocula by cocultivation on Vero E-6 cells proved to be intermediate in detection sensitivity between plaque assay and rat inoculation. Although a comparison of relative assay sensitivity was not a primary objective, this observation suggests that in vivo rat assays should be considered when maximum sensitivity is required for hantavirus detection.

None of 24 control rats seroconverted, and we concluded that intracage transmission did not occur. PRN titers were determined for 15 IFA-positive and 6 IFA-negative sera, which represented duplicate im and aerosol exposure trials for each virus. All IFA-negative sera were also negative by PRN. With the exception of 1 PUU sample that had a homologous PRN titer of 128, all other PRN titers to homologous viruses were ≥ 256 and all heterologous titers were ≤ 64.

The serological response to im inoculation of live and inactivated HTN virus is shown in Table 2. Inoculation with 1.3×10^6 or 4.3×10^6 PFU of inactivated virus elicited IFA geometric mean titers of 5.6 and 32.0, and seroconversion rates of 12.5% and 53.3%, respectively. Conversely, 2.0 PFU of live virus produced a geometric mean titer of 1,778.3 and 100% seroconversion. Since

TABLE 1

Median infectious dose values for Wistar rats after aerosol or intramuscular exposure to Hantaan, Seoul, and Puumala viruses*

Route of exposure	Virus	PFU ID_{50}
Aerosol	Hantaan	0.5 (0.3–1.1)†
	Seoul	0.7 (0.3–1.5)
	Puumala	0.3 (0.1–0.4)
Intramuscular	Hantaan	0.007 (0.002–0.014)
	Seoul	0.003 (0.002–0.005)
	Puumala	0.016 (0.005–0.049)

* Values represent combined data from duplicate trials for each virus and route of exposure.
† 95% confidence interval.

TABLE 2

Serological response to intramuscular inoculation of inactivated and live Hantaan virus

Virus	Dose (PFU)	Sample size	IFA geometric mean titer	Percent seroconversions
Inactivated*	1.3×10^6	8	6	13
	4.3×10^6	15	32	53
Live	2.0×10^{-2}	10	219	80
	2.0	10	1,778	100

* Titer prior to BPL and cobalt 60 inactivation.

we demonstrated 100% seroconversion in animals receiving <100 PFU by aerosol (Fig. 1), it is likely they experienced active infection.

DISCUSSION

The results clearly demonstrate the susceptibility of Wistar rats to infection with aerosolized hantaviruses. Although this study did not simulate realistic environmental conditions, infection occurred after aerosol exposure to extremely small amounts of virus delivered at atmospheric pressure. Intramuscular exposure to live virus was manifested by notably higher antibody titers and seroconversion rates than exposure to larger doses of killed virus. The serological response to aerosol also signifies that viral replication, not immunization by aerosolized antigen, was responsible for the IFA titers observed. Furthermore, all 3 hantaviruses produced similar dose-response curves and overlapping ID_{50} confidence intervals when administered by the same route. We conclude from these results, and previously published epidemiological and laboratory observations, that infection via aerosolized hantaviruses is a likely route of infection for rodents and humans.

The demonstration of greater rat sensitivity to im exposure than to aerosol exposure was especially noteworthy in light of recent findings by other investigators. Ecological studies of free-living rats in Baltimore, Maryland, revealed a high incidence of wounding among both sexes, which increased in frequency and severity with age and body mass.[20] Hantaviral antibody prevalence rates among these rats increased similarly, suggesting that wounding may be a factor in hantavirus transmission.[21] H. W. Lee recently showed that im infection of Sprague-Dawley rats with various strains of SEO virus resulted in consistent virus recovery from saliva (personal communication). The extreme sensitivity of rats to im-inoculated hantaviruses and the presence of infectious virus in saliva suggest that wounding may also be an important route of viral transmission among rodents.

Although im transmission may play an important role in wild virus maintenance, infection of humans is considered more likely by the aerosol route. Even though isolated cases of human HFRS are bite-related, many reported cases lack this association. Lee et al. reported more than 2,400 cases of KHF and 10%–15% mortality among United Nations troops during the Korean conflict,[22] and Powell reported 1,016 cases and 80 fatalities in 1951 alone.[23] Van Der Groen[24] cited personal communications with Song, who reported 130,000 hospitalized cases and 5% mortality in China from 1980–1982, and with Lee, who reported 400–800 hospitalized Korean cases yearly in 1984. Disease incidence this high implies a route of infection other than by biting, which suggests a direct human-rodent encounter for each case. We believe this study provides the first conclusive evidence of hantavirus aerosol transmission, confirming the suspicions of previous investigators who proposed this route of transmission for hantaviruses.

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